Molecular markers provide an independent test of species boundaries in the two morphologically similar species *Desmocladus flexuosus* and *D. asper* (Restionaceae)

Elizabeth A. Sinclair[1,2] and Russell L. Barrett[2,3,4]

[1]Division of Science and Engineering, Murdoch University, Murdoch, Western Australia 6150
[2]Botanic Gardens Park Authority, Kings Park and Botanic Garden, Fraser Ave, West Perth, Western Australia, 6005
[3]School of Plant Biology, The University of Western Australia, Crawley, Western Australia 6009
[4]c/- Western Australian Herbarium, Department of Environment and Conservation, Locked Bag 104, Bentley Delivery Centre, Western Australia 6983

Abstract

Sinclair, E.A. & Barrett, R.L. Molecular markers provide an independent test of species boundaries in the two morphologically similar species *Desmocladus flexuosus* and *D. asper* (Restionaceae). *Nuytsia* 20: 7–17 (2010). *Desmocladus flexuosus* exhibits extensive morphological variation across its geographic range, and even within the Perth metropolitan area. It may be potentially confused with its congener, *D. asper,* across this region where the two are sympatric. Here we use molecular markers to show that these two species are genetically distinct, and describe several morphological characters that can aid identification in the field.

Introduction

Restionaceae are a large clade of grass-like or sedge-like rushes, containing approximately 490 described species (Linder *et al.* 1998). A combination of few morphological characters and significant variation in intraspecific growth form (depending on age of the plant and environmental conditions) make it difficult to differentiate between some species in the field, particularly in the absence of flower spikelets.

Genus level phylogenies for the Restionaceae, including Australian members, have been published based on morphological (Linder *et al.* 2000) and molecular data (Briggs *et al.* 2000, Linder *et al.* 2003; Hardy and Linder 2005). However, for Australian taxa relationships below the level of genus have not been examined in any detail with molecular tools.

Desmocladus flexuosus (R.Br.) B.G. Briggs & L.A.S. Johnson is widespread in the coastal regions of Western Australia between Kalbarri and Israelite Bay, and is broadly sympatric with *D. asper* (Nees) B.G. Briggs & L.A.S. Johnson across large parts of that region, including the Perth metropolitan area (Figure 1). Plants are dioecious, with individual rhizomes giving rise to many branching culms up to 30–40 cm in height. *Desmocladus flexuosus* is similar morphologically to *D. virgatus* and *D. austrinus*, though their tufted habit makes them readily recognisable, especially in the field (Briggs and Johnson 2001). These two species are absent from the Perth area, making *D. flexuosus* and *D. asper* the closest

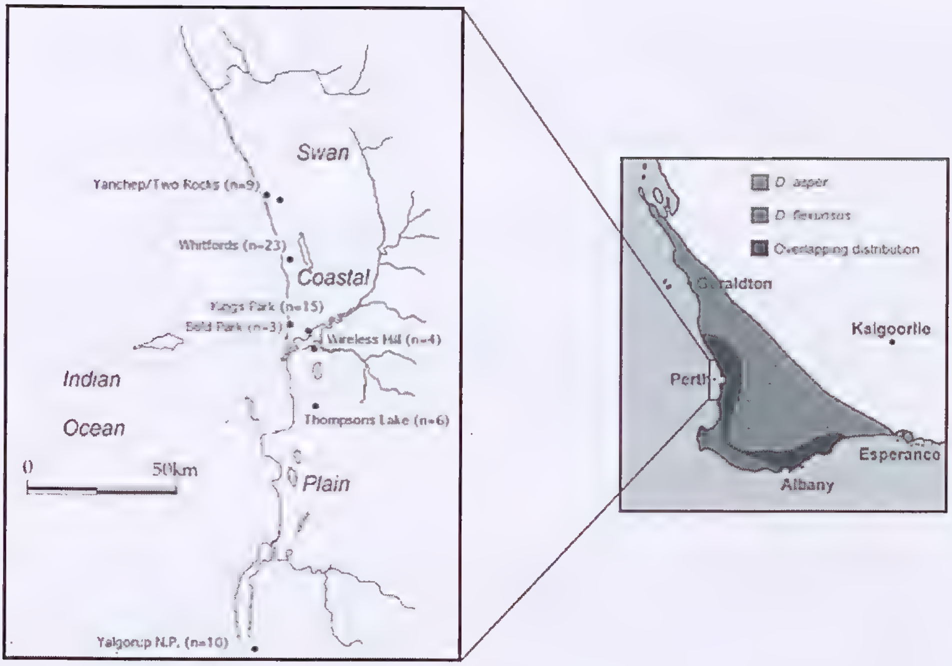
Swan
Coastal
Plain
Indian
Ocean
Yanchep/Two Rocks (n=9)
Whitfords (n=23)
Kings Park (n=15)
Bold Park (n=3)
Wireless Hill (n=4)
Thompsons Lake (n=6)
Yalgorup N.P. (n=10)
0
50km
D. asper
D. flexuosus
Overlapping distribution
Geraldton
Kalgoorlie
Perth
Esperance
Albany

Figure 1. Map showing the distributions of *D. asper* and *D. flexuosus* in Western Australia (modified from *Florabase*: http://florabase.dec.wa.gov.au/). Note the significant area of overlap between the two species. Enlargement shows the sampling locations for *Desmocladus* spp. Sample sizes are given in parentheses.

relatives in this region. *Desmocladus asper* can be distinguished by its rough-tuberculate culms and sessile male spikelets. However, *D. flexuosus* and *D. asper* are difficult to differentiate when sterile. While neither species is listed as threatened, they are important species in the stabilization of soils, and may be required in bushland restoration projects. Here, we generate genetic markers and collect morphological measurements to (1) determine whether *D. flexuosus* and *D. asper* can be differentiated using genetic markers, and (2) assess morphological characters that may allow accurate identification of sterile specimens in the field.

Materials and Methods

Sampling

Fresh whole culms were sampled for genetic analysis using Amplified Fragment Length Polymorphic loci (AFLPs). The location of samples was recorded by GPS. Culms were collected between July and November 2004 with sample sizes as follows: Bold Park (n=3), Yanchep National Park/Two Rocks (n=9), Whitfords (n=23), Wireless Hill (n=4), Kings Park (n=15), Yalgorup National Park (n=10), and Thompsons Lake (n=6) (Figure 1). Culms were collected at a minimum distance of 5 m to avoid sampling the same plant. Vouchers for all reference material are deposited in the Kings Park and Botanic Gardens (KPBG) Herbarium.

Molecular methods

Genomic DNA was extracted using the plant Qiagen kit (Qiagen Inc.). Extractions were performed on material from a single culm, with approximately 0.2–0.4 g plant material ground in liquid nitrogen prior to extraction. AFLPs are dominant multilocus markers (Vos *et al.* 1995). AFLP fingerprint profiles were generated for each sample. The restriction enzymes used were *Eco*RI and *Mse*I. Primer sequences for preselective PCR were (5' to 3') GACTGCGTACCAATTCA and GATGAGTCCTGAGTAAC. An additional two bases were added to the 3' end for selective PCR primers. We used three sets of primers: m-CTT/e-act (6-Fam label), m-CTT/e-agg (Vic label), and m-CTT/e-acc (Ned label) (Table 1). Standard protocols were followed, as described in Zawko *et al.* (2001). Bands were visualized using an ABI 377 sequencer and GENESCAN software (Applied Biosystems) with internal size standard (GS-500 ROX; Applied Biosystems). We imported GENESCAN files into Genotyper (Applied Biosystems) and each DNA profile was scored for the presence (1) or absence (0) of bands between 100 and 450 base pairs. The reproducibility of bands was assessed using five samples, each with two separate DNA extractions. A Principal Coordinates Analysis (PCA) was performed using GenAlEx v5 (Peakall & Smouse 2001) to show the relative similarities of individuals. An Analysis of Molecular Variance (AMOVA; Excoffier *et al.* 1992) was performed with the GenAlEx program to determine the proportion of variation attributed within and between each species.

Morphological Methods

Culm morphology was examined using a light microscope. Hair type and culm texture were described following the terminology of McCusker (1999). Specific characters examined in detail were culm indumentum, culm texture, primary culm length and width, and bract dimensions (Figures 4C, 5E). These characters were chosen, as they are available in the field regardless of season or sex of the clone being examined. Measurements of culm internode length and width and bract length and width were made from five to six internode sections for each sample. These measures were plotted in two dimensional plots. Means and standard deviation were determined for each character. Representative material of differing age and ecotypes is required to fully document the variation in several of these characters, particularly indumentum.

Results

Molecular

222 AFLP bands were scored for a total of 69 samples of *Desmocladus* from seven locations for three primer pairs (Table 1). The PCA showed two distinct clusters of samples, representing the two morphologically similar species, *D. flexuosus* and *D. asper* (Figure 2). Cluster 1 (n=43; on the left) is *D. flexuosus* and Cluster 2 (n=26; on the right) is *D. asper*. The bands were highly polymorphic within both species (Table 1). Unique haplotypes (or DNA profiles) were identified for all samples. Analysis of molecular variance (AMOVA) showed that most of the genetic variation was attributed to within (90.0% and 94.0%) relative to among populations (10.0% and 6.0%, *D. flexuosus* and *D. asper* respectively).

Table 1. Summary of genetic variation by species

Primer pairs	***D. flexuosus* (n=43)**			***D. asper* (n=26)**		
	No. bands	Poly. bands	%poly bands	No. bands	Poly. bands	%poly bands
m-CTT/e-agg	39	36	92.3	50	49	98.0
m-CTT/e-acc	53	50	94.3	64	64	100.0
m-CTT/e-act	71	68	95.8	71	68	95.8
Total	163	154	94.5	185	181	97.8

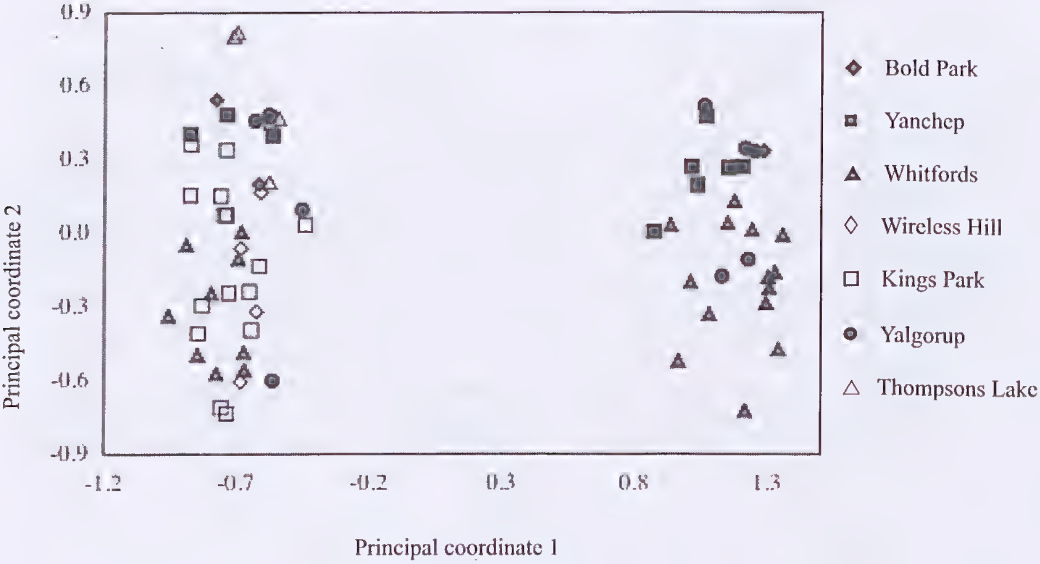

Figure 2. Principal coordinate analysis of *Desmocladus* spp. based on 222 AFLP markers. Left cluster – *D. flexuosus*; right cluster – *D. asper*.

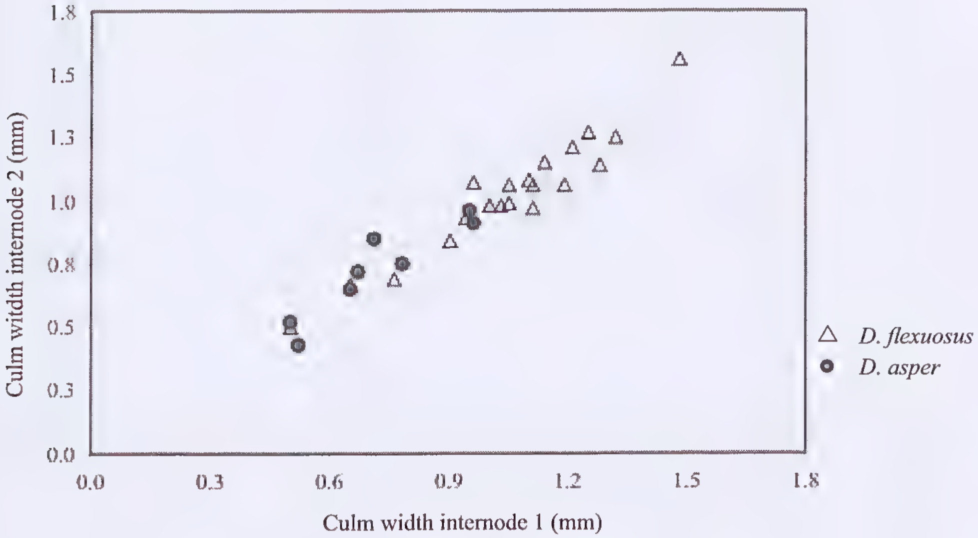

Figure 3. Plot of culm internode width for *Desmocladus asper* and *D. flexuosus*. The width of the basal culm internode is plotted against the width of the second culm internode.

Morphological

Both species were found to have a high degree of similarity, with considerable overlap in the dimensions of all characters. The range of variation in culm width is shown in Figure 3. Both taxa show partial exclusivity in their dimensions, however, the extremes of both taxa also result in considerable overlap, so that a given individual may be placed in either taxon if its culm width falls in the middle of the range of total variation. This pattern is repeated with culm length and bract dimensions (not shown). Calculation of means and standard deviations for measurements of bracts (Table 2) and culms (Table 3) shows that while the average lengths differ, the differences are not significant. Pate and Meney (1999) considered culm width to be a useful character for separating the two species, describing them as 0.7–1.2 mm wide for *D. asper* and 1.0–3.0 mm wide for *D. flexuosus*. While we found the average culm width to be larger in *D. flexuosus*, it was not significantly so. The one exception is bract width, where the average for *D. asper* is significantly less than *D. flexuosus* (Table 2). Further investigation is required to determine if this difference holds across the entire range for both taxa. The variation observed here represents general plasticity in morphology and cannot be solely attributed to variation across the geographic ranges of either species' due to the restricted sampling range relative to the total range of each species.

Indumentum type and culm texture were the only non-floral features that consistently separated the two taxa. Culm surface texture is best described as crowded-tuberculate in *D. asper*, with little or no space between the low rounded tubercles (Figure 4A, B; 5F), while in *D. flexuosus* it is sparsely tuberculate with the tubercles being more erect and scattered (Figure 5A, B, F). Culms of *D. flexuosus* are also finely striate (Figure 5A), while the culms of *D. asper* are smooth to finely pitted (punctate), becoming increasingly pitted with age. The culm hairs on *D. asper* mostly occur in sparse tufts of spreading to semi-erect hairs (Figure 4B), usually about 0.2–0.3 mm long, while culm hairs on *D. flexuosus* are long-villous, spreading to erect and irregularly twisted (Figure 5D), and (0.5)1.5–2.0(3.0) mm long.

Table 2. Mean bract width and length, with standard deviation (SD) for *D. flexuosus* and *D. asper*. Measurements taken from base of plant upwards at successive nodes, on primary culms only.

		Bract width (mm)						**Bract length (mm)**					
Culm node		1	2	3	4	5	6	1	2	3	4	5	6
D. flexuosus	Mean	3.30	3.36	3.26	3.18	3.02	2.79	8.86	8.78	8.36	8.16	6.83	6.50
	SD	0.62	0.67	0.66	0.69	0.79	0.62	1.97	1.80	1.71	1.88	1.60	2.10
D. asper	Mean	2.06	2.08	2.05	2.19	1.97	1.95	5.87	5.72	5.88	5.39	5.10	5.31
	SD	0.46	0.32	0.25	0.47	0.20	0.22	1.43	1.78	1.70	0.89	0.89	0.68

Table 3. Mean culm internode width and length, with standard deviation (SD) for *D. flexuosus* and *D. asper*. Measurements taken from base of plant upwards at successive internodes, on primary culms only.

		Culm internode width (mm)					**Culm internode length (mm)**				
Culm node		1	2	3	4	5	1	2	3	4	5
D. flexuosus	Mean	1.05	1.02	0.98	0.92	0.90	37.36	36.10	33.47	31.38	25.14
	SD	0.23	0.23	0.22	0.20	0.19	10.98	11.12	10.10	10.77	7.85
D. asper	Mean	0.72	0.72	0.70	0.71	0.70	23.14	25.95	28.95	28.39	26.53
	SD	0.17	0.19	0.18	0.16	0.17	5.93	6.46	5.86	3.48	4.41

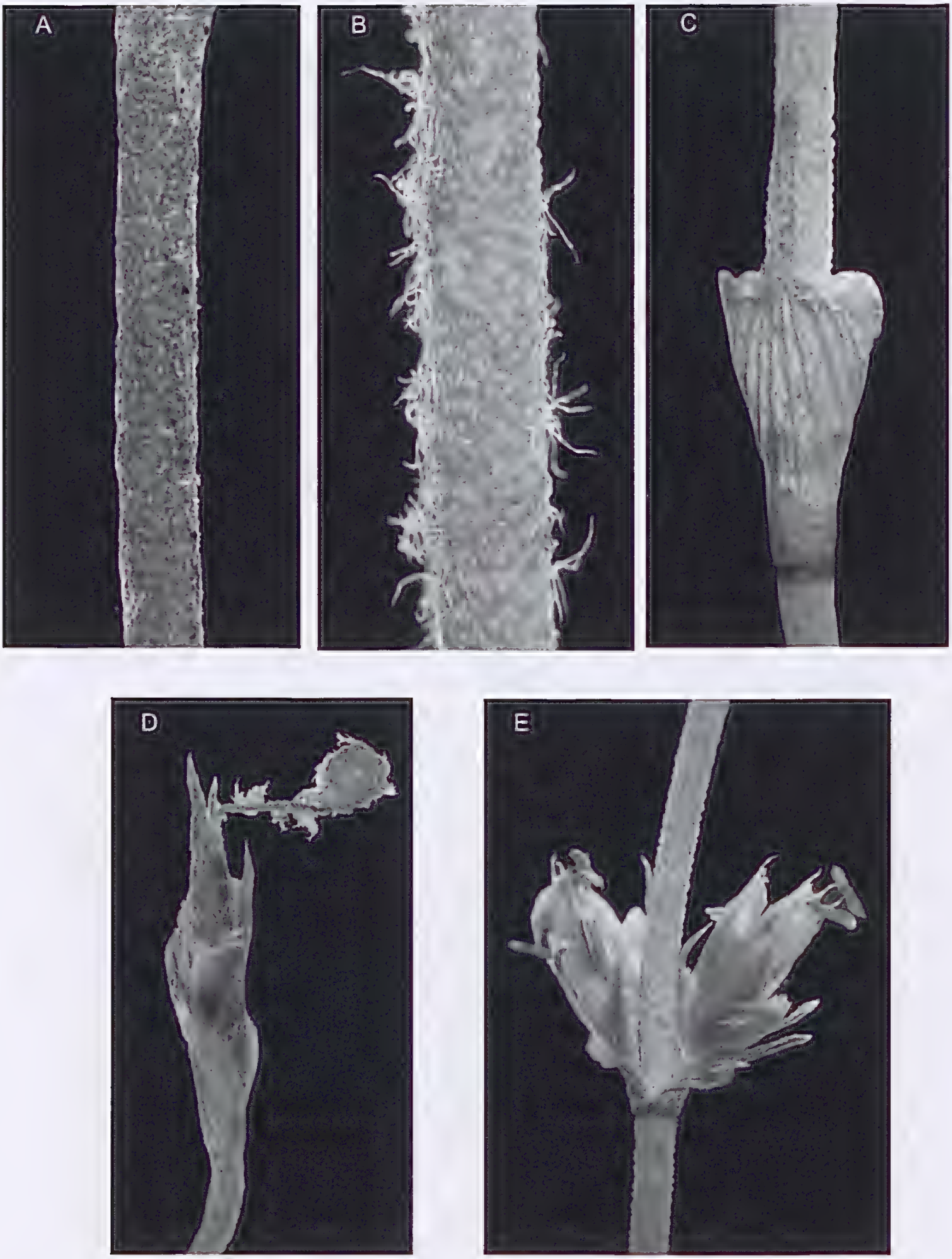

Figure 4. Morphology of *Desmocladus asper*. A – culm; B – culm with cluster hairs; C – bract; D – female spikelet; E – male spikelets. Voucher: *R.J.Cranfield* 1009/79 (PERTH).

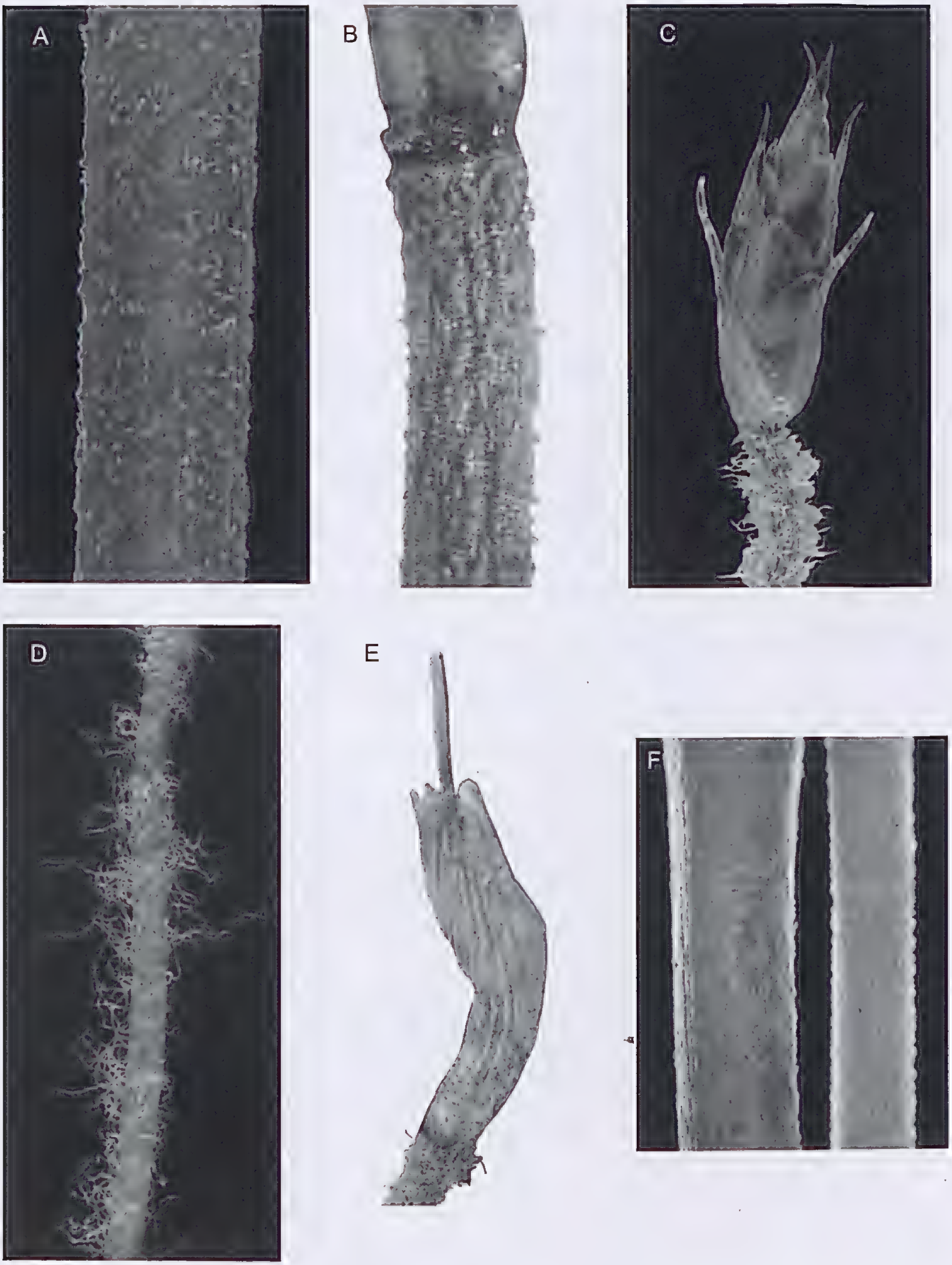

Figure 5. Morphology of *Desmocladus flexuosus*. A – culm showing striations; B – culm showing coarse striations and sparse tubercles; C – male spikelet; D – young culm showing hairs; E – bract; F. – culm of *D. flexuosus* (L) compared to culm of *D. asper* (R). Voucher: *R.D.Royce* 2589 (PERTH) (as above for F(R)).

Discussion

The genetic data showed two distinct clusters of individuals. This was in contrast to the overlapping ranges on morphological measures of culm and bract length and width. *Desmocladus flexuosus* is very similar morphologically to the sympatric *D. asper*. Examination and scoring of the vouchered material, using indumentum type and culm texture, was consistent with the genetic data and confirms their recognition as two distinct species. We are confident that these characters can be consistently scored and provide a good way to differentiate between sterile specimens of the two species in the field. In addition, data provided by B. Briggs (pers. comm. 2006) from a draft treatment of the genus for the *Flora of Australia* provide further characters considered useful in distinguishing *D. asper* from *D. flexuosus* (Table 4).

Table 4. Comparison of morphological characters of *D. asper* and *D. flexuosus* (Briggs et al. unpubl.)

Character	***D. asper***	***D. flexuosus***
Culm indumentum	Densely villous	Glabrous or villous
Hair length	Short hairs to 0.5 mm long	Hairs 0.5–2 mm long
Culm surface	Culms rough, tuberculate	Culms mostly smooth
Culm origin	Mostly arising from short rhizomes distinctly thicker than the culms (rhizomes connected by slender subterranean stems)	Arising in a tuft or ascending from an elongated subterranean portion
Male spikelets	Mostly in axils of culm sheaths, with 10–21 glumes	At ends of short branches or in axils of culm sheaths, with 4–14 glumes
Female spikelets	3.5–5 mm long	5.5–7.5 mm long

While culm indumentum is a useful character, it is also variable with plant age. In both species, hair density decreases as culms age, and both species can eventually lose all hairs (Figure 5F). The material examined from the Perth region showed a distinct difference in hair type and this difference should be examined across the range of both species. The tuberculate surface of *D. asper* is distinctive in appearance and to the touch, however, the stems of *D. flexuosus* can also be rough and somewhat tuberculate (Figure 5B). In *D. flexuosus,* the tubercles are of a different shape, appearing more peaked, and are scattered on the culms, never forming a uniform covering as in *D. asper*. Culm origin requires examination in the field and was not specifically considered in this study. The position of the male spikelets (Figure 4E) is very useful and was consistent in the specimens examined. There may also be differences in the shape of the female floral bracts (Figs. 4D, 5C), however, this requires further investigation to determine the full range of variation across the species.

Desmocladus flexuosus and *D. asper* can be readily identified by their AFLP profiles in the laboratory. It is concluded that AFLP data and morphology are congruent in indicating that *D. asper* and *D. flexuosus* are distinct species, despite considerable variation and overlap in gross morphology.

Acknowledgements

We thank M. Drobel, G. Zawko, N. Du Cros, and T. He for help in the field; R. Taylor and G. Zawko for advice and technical support; B. Briggs and A. Marchant (RBG, Sydney) for constructive discussions on the manuscript and for supplying unpublished data on these species. A. Williams at the Western Australian Herbarium is thanked for assistance with the microscope photography. All specimens were collected under CALM/DEC and Kings Park permits. This research was funded as part of an ARC linkage grant (LP0348958).

References

Briggs, B.G. & Johnson, L.A.S. (2001). The genus *Desmocladus* (Restionaceae) and new species from the south of Western Australia and South Australia. *Telopea* 9: 227–245.

Briggs, B.G., Marchant, A.D., Gilmore, S. & Porter, C.L. (2000). A molecular phylogeny of Restionaceae and allies. *In*: Wilson, K.L. & Morrison, D.A. (eds) *Monocots: systematics and evolution.* pp. 661–671 (CSIRO: Melbourne.)

Excoffier, L., Smouse, P.E. & Quattro, J.M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* 131: 179–191.

Hardy, C.R. & Linder, H.P. (2005). Intraspecific variability and timing in ancestral ecology reconstruction: a test case from the Cape flora. *Systematic Biology* 54: 299–316.

Linder, H.P., Briggs, B.G., & Johnson, L.A.S. (1998). Restionaceae *In*: Kubitski, K. (ed.) *The families and genera of vascular plants IV. Flowering plants - monocotyledons.* pp. 425-445. (Springer Verlag: Berlin.)

Linder, H.P., Briggs, B.G. & Johnson, L.A.S. (2000). Restionaceae: a morphological phylogeny *In*: Wilson, K.L. & Morrison, D.A. (eds.) *Monocots: systematics and evolution.* pp. 653–660. (CSIRO: Melbourne.)

Linder, H.P., Eldenas, P. & Briggs, B.G. (2003). Contrasting patterns of radiation in African and Australian Restionaceae. *Evolution* 57: 2688-2702.

McCusker, A. (1999). Glossary. *In*: Orchard, A.E. (ed.) *Flora of Australia.* Volume 1. 2nd ed. pp. 585 636. (ABRS/CSIRO Publishing: Melbourne.)

Peakall, R. & Smouse, P.E. (2001). *GenAlEx V5: genetic analysis in Excel: population genetic software for teaching and research.* (Australian National University: Canberra.) http://www.anu.edu.au/BoZo/GenAlEx/

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Vandelee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. & Zabeau, M. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* 23: 4407–4414.

Zawko, G., Krauss, S.L., Dixon, K.W. & Sivasithamparam, K. (2001). Conservation genetics of the rare and endangered *Leucopogon obtectus* (Ericaceae). *Molecular Ecology* 10: 2389–2396.